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Metagenomics Shows That Low-Energy Anaerobic–Aerobic Treatment Reactors Reduce Antibiotic Resistance Gene Levels from Domestic Wastewater

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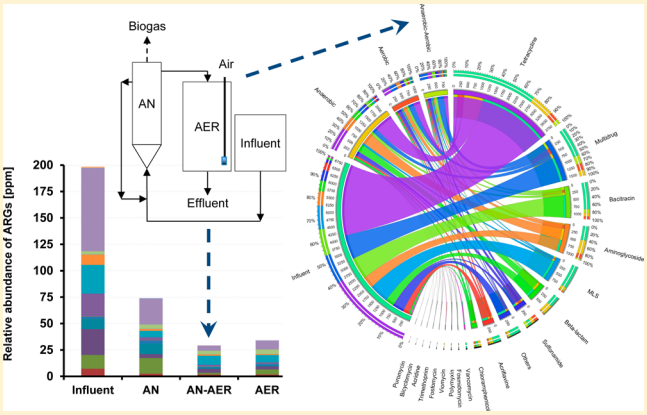
Supporting Information

ABSTRACT: Effective domestic wastewater treatment is among our primary defenses against the dissemination of infectious waterborne disease. However, reducing the amount of energy used in treatment processes has become essential for the future. One low-energy treatment option is anaerobic–aerobic sequence (AAS) bioreactors, which use an anaerobic pretreatment step (e.g., anaerobic hybrid reactors) to reduce carbon levels, followed by some form of aerobic treatment. Although AAS is common in warm climates, it is not known how its compares to other treatment options relative to disease transmission, including its influence on antibiotic resistance (AR) in treated effluents. Here, we used metagenomic approaches to contrast the fate of antibiotic-resistant genes (ARG) in anaerobic, aerobic, and AAS bioreactors treating domestic wastewater. Five reactor configurations were monitored for 6 months, and treatment performance, energy use, and ARG abundance and diversity were compared in influents and effluents. AAS and aerobic reactors were superior to anaerobic units in reducing ARG-like sequence abundances, with effluent ARG levels of 29, 34, and 74 ppm (198 ppm influent), respectively. AAS and aerobic systems especially reduced aminoglycoside, tetracycline, and β-lactam ARG levels relative to anaerobic units, although 63 persistent ARG subtypes were detected in effluents from all systems (of 234 assessed). Sulfonamide and chloramphenicol ARG levels were largely unaffected by treatment, whereas a broad shift from target-specific ARGs to ARGs associated with multi-drug resistance was seen across influents and effluents. AAS reactors show promise for future applications because they can reduce more ARGs for less energy (32% less energy here), but all three treatment options have limitations and need further study.



INTRODUCTION

Domestic biological wastewater treatment is among our most effective defenses against the transmission of infectious waterborne disease and poor water quality. Contemporary wastewater treatment plants (WWTPs) effectively reduce waste organic matter [as chemical oxygen demand (COD)], nitrogen (N), and fecal bacterial levels,[1] often employing aerobic microbiological processes with active aeration [e.g., activated sludge (AS)]. However, AS processes often have high operating costs because of energy-consuming aeration, which will almost certainly increase if energy prices rise in the future. This is a worldwide concern, but it is especially problematic in emerging countries, such as China and India, which have rapidly growing economies, increased urbanization, chronic shortages of energy, and higher baseline levels of endemic disease.

One possible energy-saving treatment option is anaerobic–aerobic sequence (AAS) reactors, which pretreat the wastewater anaerobically to reduce COD loads and potentially produce biogas, followed by aerobic processes that polish the anaerobic effluents to meet effluent discharge standards. Dependent upon the design, AAS systems can have lower

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capital costs (relative to conventional treatment systems), smaller footprints, lower sludge production, possible biogas production, and reduced energy use because of lower oxygen demand.[1−4] In fact, AAS treatment systems are now quite common for domestic waste treatment in warmer climate regions (e.g., Brazil, Egypt, India, and Israel),[5−7] although AAS systems are not typical in temperate and colder parts of the world.

Although a shift toward low-energy waste treatment has become critical, treatment technologies must still protect against disease transmission, such as reducing pathogen levels in treated effluents. However, there is strong evidence that WWTPs are reservoirs for and can promote antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARG) in their microbial communities,[8−11] which then can be released to the environment via liquid effluents and biosolids.[8,12−15] The increased potential for ARBs/ARGs in WWTPs has implications to all plants and their ability to protect against disease because acquired antibiotic resistance (AR) in pathogenic and other bacteria is increasing on a global scale.[16] Therefore, for low-energy waste treatment options to be implemented, they must be at least as effective as current treatment approaches (ideally better) in terms of ARG levels or they will not effectively protect against resistant disease dissemination.

Here, we assessed ARG dissemination in the treated effluents of three different domestic wastewater treatment options that use different amounts of energy. Specifically, anaerobic, aerobic, and AAS reactors treating domestic wastes were compared in terms of energy use, treatment performance, and ARG abundance and diversity over 6 months. High-throughput sequencing and metagenomics were employed to characterize ARG occurrence and diversity levels between influents and effluents, which were compared to relative energy consumption levels during treatment.

METHODS

Reactor Setup and Operations. Reactor configurations are shown in Figure S1 of the Supporting Information). Fresh settled wastewater was collected weekly from a local domestic WWTP in northern England and stored in 18 L carboys at 4 °C for use as reactor feeds. Mean waste influent characteristics were as follows (±standard error): soluble COD (sCOD) = 265.9 ± 14.9 mg/L, total Kejdahl nitrogen (TKN) = 82.7 ± 8.41 mg of N/L, ammonia (NH_3) = 47.2 ± 3.57 mg of N/L, and pH = 7.2 ± 0.02. Wastewater was fed using peristaltic pumps (Watson Marlow 520S, U.K.) directly into three initial reactors, which included an upflow anaerobic sludge blanket reactor (UASB), an anaerobic hybrid reactor (AHR), and a completely mixed aerobic reactor (AER1); all reactor designs used in domestic wastewater treatment.[17] Effluents from the AHR and UASB units were pumped into second-stage aerobic units (AER2 and AER3) for further treatment, respectively (i.e., the two AAS systems were AHR−AER2 and UASB−AER3). Two anaerobic reactor types were tested because a sub-goal was to contrast UASB versus AHR designs relative to ARG/ARB mitigation.

UASB and AHR reactors had working volumes of 1.5 L and height/diameter ratios of 4.0 (H/D). Wastewater influent was introduced at the bottom of the units, and both units were operated at 3 day hydraulic retention times (HRTs). Recirculation flow rates for the UASB and AHR reactors were 133 and 155 mL/min, respectively. A three-phase separator (gas−liquid−solid) was located at the top of each reactor to reduce washout of suspended solids and also to trap biogas in attached Tedlar sampling bags. The anaerobic units were maintained at 35 ± 1 °C by circulating warm water around their cores. pH was maintained between 6.8 and 7.2.

The three aerobic reactors had 1.04 L operating volumes. Wastewater was fed by a peristaltic pump in the aerobic units, either directly from the wastewater source (AER1) or from AHR (AER2) or UASB (AER3) reactor effluent lines. The aerobic reactors were operated at 2 day HRTs, which were chosen based on preliminary system testing. No recycle was provided because it was desired to keep operations simple to minimize the chance of mechanical or other failures disturbing the systems. Air supply was tightly regulated and monitored, which was essential for energy calculations. Mixing was provided using air diffusers, stir plates, and magnetic stir bars. Aerobic reactors were maintained at room temperature (20−22 °C).

Sample Collection and Routine Sample Analysis. Wastewater influent and effluent samples from the UASB, AHR, and AER1 units and effluents from the two AAS systems (UASB−AER2 and AHR−AER3) were collected and analyzed weekly. These reactors were actually operated for over 6 months; however, sample collection for metagenomic analysis was only performed over a 6 week window of time after the reactors had been very stable for 6 weeks. Our goal was to quantify ARG abundances and diversity under pseudo-steady-state operating conditions. Routine monitoring included sCOD, TKN, NH_3, and pH for all units, total suspended solids (TSS) and volatile suspended solids (VSS) for aerobic units, and methane (CH_4) production in the anaerobic units. Influent and effluent samples for DNA extraction and metagenomic analyses were collected weekly and frozen immediately.

All chemical analyses were performed according to standard methods for wastewater characterization,[18] except biogas and CH_4 analysis. Gas bag volumes were recorded regularly, and CH_4 levels were quantified using direct injection into a Carlo Erba 5160 (U.K.) Mega gas chromatograph (GC) equipped with a flame ionization detector (FID) and a HP-PLOT Q capillary column (30 m × 0.32 mm internal diameter) packed with 20 μm Q phase. The GC oven temperature was maintained at 35 °C, and the injector and detector were kept at 300 °C. Hydrogen was used as the carrier gas (1 mL/min, 65 kPa). Methane analysis standards were prepared using a 70:30% analytical-grade CH_4/CO_2 standard gas mixture (BOC Gases, U.K.). All GC injections were performed in duplicate.

DNA Extraction, Sequencing, and Bioinformatic Analysis. DNA was extracted from the weekly samples after completion of all sampling, using the Fast Soil DNA extraction kit (MP Biomedicals, Santa Ana, CA) and a Ribolyzer (MP Biomedicals, Santa Ana, CA) according to instructions by the manufacturer. After extraction, DNA were sorted and combined into two tri-weekly groups for sequencing (i.e., the first 3 weeks were combined, and the second 3 weeks were combined). Combining DNA from sequential sampling windows provided two independent samples for influent and effluent microbial metacommunities in each reactor.

To assess the diversity and relative abundances of ARGs in the combined samples, DNA extracts were provided (in duplicate) to the Beijing Genomics Institute (BGI) for shotgun library construction [insert size of 170 base pairs (bp)] and high-throughput sequencing using the Illumina HiSeq 2000 platform. Approximately 3 Gb (giga base pairs) of data was

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Table 1. Treatment and Energy Performance Data for the Aerobic, Anaerobic, and AAS Treatment Units

parameter	influent	effluents		
		aerobic	anaerobic	AAS
pH	7.19	6.53	6.73	5.96
	(0.05)[a]	(0.12)	(0.08)	(0.15)
sCOD (mg/L)	167.0	22.7	34.8	7.58
	(11.9)	(5.42)	(5.54)	(1.37)
NH_3 (mg/L)	58.9	3.22	17.3	5.84
	(1.63)	(1.47)	(1.28)	(0.77)
TKN (mg/L)	67.7	6.07	26.1	9.10
	(4.79)	(2.20)	(2.98)	(1.32)
TSS (mg/L)	ND[b]	20.2	ND	26.8
		(4.42)		(10.3)
VSS (mg/L)	ND	15.3	ND	19.3
		(5.46)		(11.9)
Energy use (KW/kg of sCOD removed)		1.9	from −0.4 to −0.7[c]	from 1.2 to 1.4

[a]Parentheses show 95% confidence intervals based on $n = 12$. [b]ND = not done. [c]Negative values indicate energy gains in the anaerobic units per COD removed.

generated for each DNA sample. Quality filtering was conducted for all metagenomic data to ensure validity in downstream analysis. Raw reads that contained three or more ambiguous nucleotides, quality scores below 20 for more than 36 bases, or with adapter contamination were removed prior to subsequent data processing. Quality-filtered metagenomic data were searched for putative ARGs against the clean antibiotic resistance database (ARDB), which had non-redundant sequences using BLASTX with an E value of $\leq 10^{-5}$.[19,20] A read was identified as an ARG-like sequence according to its best BLASTX hit with amino acid identity of ≥90% and alignment length of ≥25 amino acids.[21,22]

Identified ARG-like sequences were sorted into ARG types and subtypes, using a structured ARDB and customized python script.[20] ARG definitions, which included presumed resistance mechanisms for each ARG, were based on previous work and are simplifications because ARGs often code for proteins with multiple effects.[20,22] However, simplification is reasonable because over 230 ARGs were assessed and overarching trends were more important than individual details. To compare ARG levels among samples, the number of ARG-like sequences was normalized to the number of metagenome and ARG sequences in each sample. As such, ARG levels are reported as either "total metagenome sequences" (in ppm; one read in one million reads) or "total ARG-like sequences" as percentages (%).

Energy Calculations. Energy use was calculated for all systems, which included energy used for aeration, mixing, heating, and pumping, and potential energy gained from biogas production.[2] To produce realistic energy estimates, calculations for each case were based on extrapolated energy use in geometrically identical, scaled-up treatment units treating 1000 L/day wastewater. However, the air supply rate, mixing, and pumping rate data were based on measured lab reactor data.

Energy used in the aerated units was dominated by aeration and mixing needs, which was calculated using measured air flow rate data and equivalent paddle-mixing requirements for scaled-up aerobic reactors. Power ratings for characteristic industrial-scale centrifugal pumps were used to estimate feed and effluent recycle pump energy needs.[2] Energy estimates for anaerobic units were based on the energy required for mixing as well as the energy needed for heating anaerobic units to 35 °C. Potential energy recovery from biogas production was estimated based on previous CH_4 data and the use of industrial-scale gas turbines. Net energy used by the anaerobic units was the difference between energy used for pumping, heating, and mixing minus the potential energy gained from biogas production. Energy calculations for AAS systems included the energy used for mixing and aeration in the aerobic units (less air was used because of lower sCODs) and mixing, heating, and biogas production in the anaerobic units, which varied slightly between the UASB and AHR reactors.

Other Data Analysis. This study contrasted aerobic, anaerobic, and AAS treatment systems relative to ARG fate and energy use using two independent influent and effluent metagenomes per system (except AHR, where one metagenome was available; see Tables S1−S3 of the SI). However, to allow for statistical comparisons among the three treatment options, it was first necessary to determine whether ARG characteristics between each duplicate pair were statistically the same in paired DNA samples. One-way analysis of variation (ANOVA) and t tests were performed on relative ARG levels in each duplicate for the 19 AR groups (identified in the metagenomic analysis; see Table S1 of the SI). If the duplicate pairs were statistically the same (i.e., $p < 0.05$), data from the duplicates were clumped as averages to allow for more rigorous statistical comparisons among treatment opens. Follow-on tests included two-sample testing, such as ANOVA among reactors under each treatment, and statistical comparisons between performance data for each reactor system. All analyses were conducted using SPSS (version 17.0, Chicago, IL). The Venn diagram was plotted using Venny (http://bioinfogp.cnb.csic.es/tools/venny/), and the relatedness of influents and treated effluents was visualized using Circos.[23]

RESULTS AND DISCUSSION

Bioreactor Performance, Operational Data, and Energy Used Per Design. Two anaerobic (UASB and AHR), one aerobic (AER1), and two AAS (AHR−AER2 and UASB−AER3) treatment systems (see Figure S1 of the SI) were monitored to compare treatment performance and ARG metagenomes before and after treatment of domestic wastes. Relative to treatment performance, Table 1 data show that effluent sCOD, TKN, and NH_3 levels did not significantly differ between the two anaerobic reactors or between the two AAS systems ($p > 0.05$). Therefore, these data were grouped under "anaerobic" (UASB and AHR combined) and "AAS" (AHR−

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AER2 and UASB−AER3 combined), which were then statistically compared to each other and the "aerobic" treatment unit (AER1), relative to effluent sCOD, TKN, and NH_3 levels.

The AAS systems had significantly lower effluent sCOD levels than aerobic or anaerobic units alone ($p < 0.027$ and $p < 0.002$, respectively). In contrast, aerobic units had significantly lower effluent TKN and NH_3 levels than the AAS systems ($p < 0.024$ and $p < 0.028$, respectively), but the AAS systems had significantly lower TKN and NH_3 effluent levels than anaerobic units alone ($p < 0.002$ and $p < 0.002$, respectively). Therefore, sCOD and N removal levels were superior in the aerobic and AAS systems to the anaerobic units alone, which is consistent with past studies on aerobic and AAS systems in domestic wastewater treatment, especially sCOD removals.[3]

AAS systems used ~32% less energy (per kilogram of sCOD removed) relative to the aerobic units (Table 1), largely because of lower air use in the AAS aerobic units. Biogas production itself (i.e., CH_4) was low and erratic in both anaerobic reactors, typically less than 10% CH_4 in the gas collectors. Therefore, potential energy gains from biogas production were comparatively small in our AAS and anaerobic systems (Table 1). In reality, low levels of biogas production is a common problem in the anaerobic treatment of relatively dilute domestic wastes[3] and is partly why it is not used in many places. Regardless, the AAS systems show promise because they attain similar effluent quality to aerobic units, require less energy, and produce far superior quality effluents to anaerobic units. However, effluent ARG characteristics from AAS systems must be determined relative to aerobic and anaerobic alone systems.

Total Abundance of ARGs. Core ARG metagenomic data for the reactors are summarized in Tables S1−S3 of the Supporting Information. For statistical purposes, the absolute number of detected ARG-like sequence reads were first normalized to total metagenomic sequences to avoid bias caused by different sequencing depths among the samples. On the basis of normalized data (see Table S1 of the Supporting Information), one-way ANOVA analysis of the three influent samples ($p = 0.82$), the three anaerobic effluent samples (two UASB and one AHR; $p = 0.82$), the four AAS effluent samples ($p = 0.86$), and the two aerobic effluent samples ($p = 0.84$) was performed and no statistically significant differences were seen among samples within each grouping. As such, subsequent analysis comparing influent and anaerobic, aerobic, and AAS effluent metagenomes used grouped data to improve statistical significance.

Normalized ARG abundances decreased through treatment relative to influent ARG levels for all three treatment options (see Figure 1A). The mean influent ARG abundance was 198 ppm, whereas anaerobic, aerobic, and AAS effluents had 74, 34, and 29 ppm ARG abundances, respectively (62.6, 82.8, and 85.3% reductions); i.e., ARG abundances were over double in anaerobic effluents compared to aerobic and AAS effluents. However, Figure 1B also shows some ARG types proportionally increased after treatment, including ARGs presumed to be associated with multi-drug resistance, especially in the AAS and aerobic effluents.

Results are generally consistent with previous work.[10,12,24−27] Wastewater treatment can reduce apparent AR levels, although removal efficiencies vary depending upon reactor design, operating conditions, and ARG/ARBs assessed. Various treatment systems have been assessed relative to ARG and ARB fate, including AS, biofilters (BFs), and submerged aerated

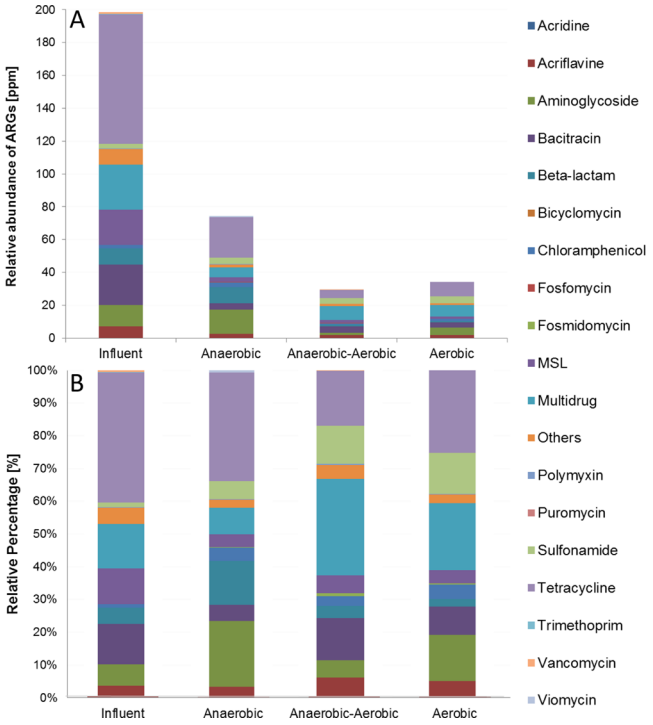

Figure 1. Relative abundance of ARGs in the influent and effluent samples. (A) Relative abundance of ARG-like sequences normalized to the total number of reads in the metagenome. (B) Relative percentages of different ARG types of the influent and effluent samples. MLS denotes macrolide−lincosamide−streptogramin. Others represents resistance genes that are not directly related to specific antibiotic classes.

filters (SAFs). Although it is hard to compare results from different studies (because detection methods differ), general observations are possible. As an example, the influence of plant size and operating conditions was assessed on removal of amoxicillin-, tetracycline-, and ciprofloxacin-resistant heterotrophic bacteria, enterobacteria, and enterococci.[25] SAF and AS systems efficiently reduced total bacteria, although removals did not correlate with ARB removal rates in the systems. In contrast, total bacterial removal rates were lower in BFs, which displayed greater reductions in ARB levels relative to SAF and AS designs.

Anaerobic treatment systems were a part of a large study of northern Chinese WWTPs, which quantified bla_{NDM-1} gene levels across two full-scale treatment plants.[10] The authors found bla_{NDM-1} gene numbers increased from the primary clarifier through two biological treatment steps (anoxic and then aerobic tanks in both WWTPs), confirming that ARG abundances can increase in some unit operations. Interestingly, only after biosolids were removed by secondary clarification did bla_{NDM-1} levels decline in their systems, implying that bla_{NDM-1} is associated with the sludge biomass; i.e., genes are moving with the biosolids, not necessarily being destroyed. Clearly, treatment processes can either increase or decrease ARG and ARB abundances relative to wastewater influents depending upon the ARGs studied and treatment conditions.

ARG Diversity and Resistant Types among Reactor Influent and Effluents. A total of 19 ARG types (see Tables S1 and S2 of the Supporting Information) were assessed in samples using metagenomics and deep sequencing methods, which were further classified into ARG subtypes (see Table S3

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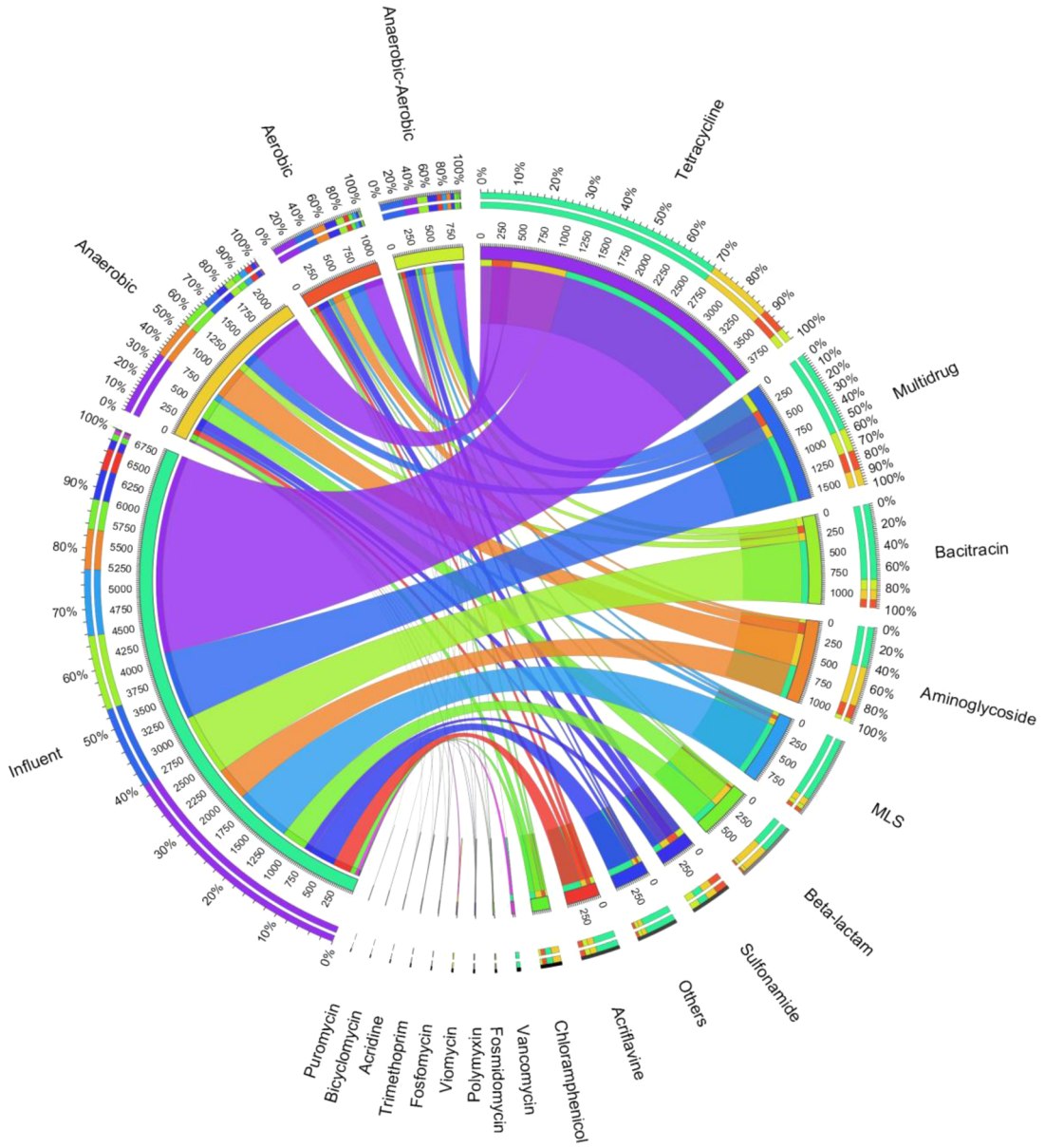

Figure 2. Distributions of ARG types in total annotated ARG sequences in the influent and effluents (anaerobic, aerobic, and anaerobic−aerobic). The data were visualized using Circos.[22] The length of the bars on the outer ring represented the percentage of ARGs in each sample (left side of the diagram) and correlates the percentages of respective ARG types in the influent, aerobic, anaerobic, and anaerobic−aerobic samples (right side of the diagram). MLS denotes macrolide−lincosamide−streptogramin. Others represents resistance genes that are not directly related to specific antibiotic classes.

of the Supporting Information). The most prominent ARG types found in wastewater samples were tetracycline, sulfonamide, multi-drug resistance, macrolide−lincosamide−streptogramin (MLS), chloramphenicol, β-lactam, bacitracin, aminoglycoside, and acriflavine (Figure 2). Overall abundances of ARG sequences were lower in effluent versus influent samples based on ARG sequence/absolute read data, although percentage changes differed significantly between ARG type and treatment conditions (see Table S2 of the Supporting Information). A total of 17 of the 19 ARG types were detected in influent samples, with dominant types being tetracycline (39.7%), multi-drug (13.7%), bacitracin (12.3%), and then MLS (10.8%) (see Table S2 of the Supporting Information). Similarly, 17 of 19 ARG types were detected in anaerobic effluents, with tetracycline (33.2%), aminoglycoside (20.1%), β-lactam (13.5%), and multi-drug (8.2%) being dominant. In contrast, only 13 ARG types were found in aerobic effluents, with dominant ARGs being tetracycline (25.2%), multi-drug (20.6%), aminoglycoside (14%), and sulfonamide (12.6%). Finally, 16 ARG types were found in AAS effluents, with tetracycline (16.8%), sulfonamide (11.6%), multi-drug (29.4%), and bacitracin (13%) resistance being the most common.

Various observations are possible from Figures 1 and 2. First, three ARG types were consistently higher in anaerobic effluents relative to the AAS and aerobic effluents (in ppm): tetracycline, aminoglycoside, and β-lactam sequences. This implies the ARG types may more readily migrate through anaerobic versus aerobic treatment processes, although there is no simple mechanistic explanation for this phenomena. Second, neither sulfonamide nor chloramphenicol ARGs were reduced by any

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treatment option, suggesting that ARGs for these antibiotics may not be removed by any biotreatment process, which was also seen in Swiss and Hong Kong WWTPs.[20,28] Finally, percentage levels of some ARG types were higher after treatment, most noteworthy being multi-drug resistance ARGs in aerobic and AAS effluents. Increases in multi-drug resistance has been seen previously in WWTP processes,[29] but data here imply that relative increases may be greater in processes that include aerobic steps. This observation is consistent with data from Yang et al., who observed elevated aminoglycoside, tetracycline, sulfonamide, and multi-drug resistance ARGs in aerobic AS samples from the Shatin WWTP in Hong Kong.[20] Zhang et al. also detected elevated tetracycline, macrolide, and multi-drug resistance ARG sequences in mobile metagenome studies on AS, suggesting that associated resistance determinants are on mobile genetic elements (MGEs) within the wastes.[22] Despite these observations, it is key to note that observed increases are percentage values based on many fewer ARG sequence reads in effluent samples. Absolute levels of multi-drug resistance ARGs actually declined in treated effluents relative to influent levels.

ARG Subtypes in the Different Reactor Effluents. Although patterns of ARG transmission in different treated effluents can be inferred from ARG type data, additional information can be gained from ARG subtype data. Influent samples had the highest abundance of ARG subtypes (219 of 234 ARG subtypes assessed) and reflected almost all ARG subtypes seen in reactor effluents (Figure 3 and Table S3 of the Supporting Information).[30,31] The most abundant ARG subtypes in the influent were related to tetracycline and bacitracin, i.e., *tet*M (14 ppm), *tet*A(39) (12 ppm), *tet*W (12 ppm), *tet*32 (12 ppm), *bac*A (11 ppm), and *upp*P (11 ppm), which are among the most common forms of resistance found in the human gut and fecal DNA.[32−34] In contrast, *sul*1, which confers sulfonamide resistance, was abundant in all effluents (2.4, 1.9, and 3.3 ppm in aerobic, AAS, and anaerobic effluents, respectively). Other notable ARG subtypes seen in the effluents were HAE1, the multi-drug efflux gene, found in aerobic (2.4 ppm) and AAS (2.1 ppm) effluents, *tet*A (5.2 ppm) in aerobic effluents, and *upp*P (2.3 ppm) found in AAS effluent. Tetracycline resistance gene *tet*V (21 ppm), aminoglycoside 2-*N*-acetyltransferase (12 ppm), and β-lactam-β-lactamase (8 ppm) were abundant in anaerobic effluents.

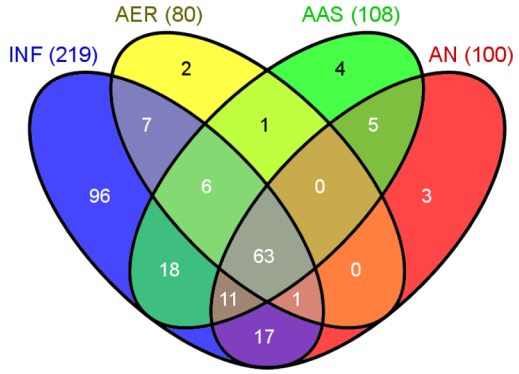

Figure 3. Venn diagram of ARG subtypes found in the influent (INF) and the effluents from the anaerobic (AN), aerobic (AER), and anaerobic−aerobic sequence (AAS) reactors. These equate to 137 ARG subtypes in the effluents compared to 219 ARG subtypes found in the wastewater influent.

While subtype ARG abundances declined through treatment, there were still 63 "persistent" ARG subtypes detected across samples (including influents), which included 14 multi-drug resistance, 10 aminoglycoside, 9 tetracycline, 7 MLS, and both sulfonamide ARG subtypes (Figure 3). Disturbingly, these 63 subtypes accounted for 75, 98, 95, and 94% of the total relative abundance of ARGs in the influent and aerobic, AAS, and anaerobic effluents, respectively. Yang et al. similarly found 78 (of a total of 271) persistent subtypes in Shatin WWTP.[35] Finally, only 14 influent ARG subtypes were found in the effluents from aerobic or AAS processes, whereas 46 influent ARGs were seen in effluents from anaerobic or AAS processes (Figure 3). This suggests that the inclusion of an anaerobic step in a treatment sequence may promote a greater number of unique ARG subtypes than inclusion of aerobic steps.

ARG Resistance Mechanisms. Antibiotic resistance is caused by four primary mechanisms: (1) efflux pumps, (2) inactivation, (3) target bypass, and (4) target modification.[36−43] The dominant resistance mechanisms observed in our influent samples were efflux pump and target modification mechanisms (~38%; Figure 4). However, relative percentages of efflux pump ARGs increased, and target modification ARGs declined through all treatment options. As background, efflux pumps are membrane-associated proteins that recognize and pump or exclude antibiotics from the cell, reducing intracellular antibiotic levels, which allows protein synthesis to proceed.[39] Efflux pumps can either be specific for one compound (e.g., an antibiotic) or can transport a wide range of chemically similar compounds. Therefore, efflux pump systems often provide defense against an array of inhibitory substances, including the potential for conferring multi-drug resistance. Multi-drug efflux proteins are generally encoded on the chromosome, while drug-specific mechanisms are often encoded on MGE.[39,41,42]

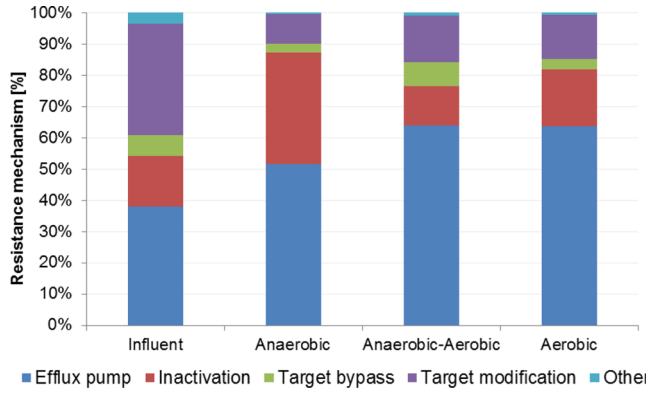

Figure 4. Detected percentages of each resistance mechanism for the influent and effluents from each treatment reactor condition. Other represents mechanisms that are not directly related to specific antibiotic classes.

Overall, Figure 4 data suggest that waste treatment selects toward efflux and away from target modification resistance mechanisms, especially in aerobic treatment processes (see Tables S3−S5 of the Supporting Information). Given that ARGs, which code for specific efflux pumps, are often found on MGEs (see the Supporting Information for further discussion),[31,39,42,43] the presence of diverse chemical stressors in waste treatment environments (e.g., heavy metals, disinfectants, or other toxins) might favor the acquisition of multiple efflux defense traits in their microbial communities. Gillings recently

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showed that bacterial stress can accelerate intracellular gene rearrangement.[44] Therefore, a shift away from specific-target modification ARG sequences[45] to both specific and multi-drug efflux ARGs (and related mechanisms) might be expected in waste treatment environments.[46]

Practical Implications. The practical question is "how do such observations relate to potential implementation of lower energy treatment technologies, such as AAS". In reality, the answer is not 100% clear. On the basis of results here, AAS and aerobic treatment systems appear to be superior to anaerobic alone processes relative to mitigating against ARG dissemination. COD and N removal rates were also better in these systems. However, results also show that AAS and aerobic systems may select for proportionally greater levels of multi-drug resistance.[15,28,29] Therefore, no current treatment option is perfect, and more work is needed at understanding how resistance is transmitted in all treatment processes, not the least of which is in biosolids,[10] which was not addressed in this study. In fact, reducing ARGs in biosolids processing may be where work is most needed to reduce overall ARG releases from WWTPs.

Our metagenomic data suggest that aerobic processes may be generally better than anaerobic processes for reducing ARG sequences through treatment and AAS can provide equivalent treatment performance and ARG reduction for less energy (~32% here). Therefore, AAS reactors show considerable promise for future waste treatment applications. However, all treatment options have limitations, and work is needed to understand ARG fate in waste treatment, especially ARGs coding for potential multi-drug resistance in treated effluents.

■ ASSOCIATED CONTENT

Supporting Information

Additional information on relative abundance of each ARG type in the respective raw samples (Table S1), percentage of each ARG type in the respective raw samples (Table S2), presence/absence of each ARG subtype in waste influent and ARG subtypes found in the respective effluents (Table S3), percentage of ARGs coding for the major antibiotic resistance mechanisms in the respective samples (Table S4), percentage of main resistance mechanisms for dominant ARGs among different antibiotic classes in the samples (Table S5), and schematic of the reactor system used in this study (Figure S1). Additional results and discussion are also provided on the role of ARGs on MGEs and the acquisition of multi-resistance among bacteria in wastewater treatment environments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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